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Ca^{2+} Affects Physicochemical and Conformational Changes of Threadfin Bream Myosin and Actin in a Setting Model

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Abstracts

The effect of Ca^{2+} on physicochemical and conformational changes of threadfin bream (TB) myosin and actin during setting at 25 and 40 °C was investigated. Ca^{2+} ion at 10-100 mM induced the unfolding of myosin and actin as evident by an increase of surface hydrophobicity (S_o ANS) at 40 °C. Total SH groups also decreased with an increased Ca^{2+} concentration, suggesting that Ca^{2+} promoted the formation of disulfide bonds during setting at 40 °C. Both hydrophobic interactions and disulfide linkages were involved in formation of myosin aggregates at 40 °C, and were enhanced by addition of 10-100 mM Ca^{2+}. Myosin Ca-ATPase activity decreased at Ca^{2+} > 50 mM, indicating conformational changes of myosin head. Circular dichroism spectra demonstrated that Ca^{2+} reduced the α-helical content of myosin and actin incubated at either 25 or 40 °C. Ca^{2+} induced conformational changes of TB myosin and actin incubated at 40 °C to a greater extent than at 25 °C.

Key words: Threadfin bream, myosin, actin, calcium, setting

Introduction

When fish muscle proteins are grounded with 2- 4 % salt and pre- incubated at 4-40 °C for a period of time prior to heating, an increase in gel elasticity is observed. Such phenomenon is known as "setting" or "suwari" in Japanese (Lanier 2000). It has been generally accepted that setting is mainly attributed from the activity of endogenous transglutaminase (TGase), the Ca^{2+}-dependent enzyme (Kumazawa and others 1995; Benjakul and others 2004). The enzyme catalyzes an acyl transfer reaction between γ-carboxy amide groups of glutamyl residues in proteins as the acyl donor and variety of primary amines as the acyl acceptor (Folk 1980). The formation of ε-(γ-glutamyl) lysyl isopeptide bonds between glutamine (acyl donors) and lysine (acyl acceptor) resulted in a covalent cross-linking of muscle proteins.

Addition of Ca^{2+} has been reported to improve textural properties of Pacific whiting, threadfin bream, and Alaska pollock surimi (Lee and Park 1998; Yongsawatdigul and others 2002). Gel enhancing effect is more evident when sample is subjected to setting. It has been typically believed that Ca^{2+} improves gel-forming ability of fish proteins by activating fish endogenous TGase (Lanier 2000). However, Ca^{2+} is also known as a destabilizing salt in the Hofmeister series (Baldwin 1996). Binding of Ca^{2+} to proteins prevents the salt exclusion, resulting in a decrease of preferential hydration and destabilized structure (Arakawa and Timasheff 1984). The effects of Ca^{2+} on structural changes of various proteins have been reported. Ca^{2+} solubilized rabbit myofibrillar proteins by salting-in effect (Taylor and Etherington 1991). Tertiary and secondary structure of α-crystallin decreased in the presence of Ca^{2+} (Valle and others 2002). Moreover, binding of Ca^{2+} to β-lactoglobulin induced partial unfolding which led

to an increased hydrophobicity during gelation (Jeyarajah and Allen 1994). Therefore, Ca^{2+} could also have a direct effect on structure of muscle proteins, which could affect gelation during setting. The role of Ca^{2+} on such conformation changes of fish protein has not been thoroughly investigated.

Ogawa and others (1995) found that the unfolding of actomyosin as measured by a decrease in α-helicity was a pre-requisite to initiate setting of actomyosin. Hydrophobic interactions were also responsible for aggregate formation of cod and herring myosin during setting at 40 °C (Gill and others 1992). In addition, formation of disulfide bonds was noticed during setting of herring myofibrillar proteins (Chan and others 1995). These studies suggested that other bondings, besides ε-(γ-glutamyl) lysyl isopeptide bonds, were involved in setting. However, the effect of Ca^{2+} on hydrophobic interactions and disulfide linkages of fish myosin and actin during setting have not been elucidated.

Threadfin bream (*Nemipterus spp.)* is the second largest resource used for surimi production, after Alaska pollock. Thailand is one of the major threadfin bream surimi producers in the world with an approximate annual production of over 80,000 metric tons. Despite of its large production quantity and value, scientific information related to setting phenomenon is still limited. Understanding the role of Ca^{2+} ion on conformational changes of myosin and actin would be critical knowledge for improving textural properties of surimi gels from threadfin bream and other warm water species. Therefore, our objectives were to investigate the effects of $CaCl_2$ on physicochemical and conformational changes of threadfin bream myosin and actin during incubated at 25 and 40 °C, typical setting temperatures of fish proteins.

Materials and Methods

Fish sample

Threadfin breams (TB) (*Nemipterus bleekeri*) were caught off the Gulf of Thailand at Rayong province. Fish were immediately transported to a Suranaree University of Technology laboratory in polystyrene boxes packed with ice. Fish were manually eviscerated upon arrival and kept on ice. Myosin preparation was carried out 24 h after catch.

Myosin Preparation

Myosin was purified according to the method of Martone and others (1986) with slight modifications. All steps were performed at 0-4 °C to minimize proteolysis and protein denaturation. TB mince was added with 10 volumes of buffer A (0.10 M KCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.02 % NaN_3 and 20 mm Tris-HCl, pH7.5) and homogenized at 15,000 rpm for 2 min in a homogenizer (AM-8, Nihonseiki Kaisha, Ltd., Tokyo, Japan). The homogenate was stirred for 10 min and centrifuged at 1,000×g (Sorvall RC-5C Plus, Dupont, Del., USA) for 10 min. The pellet was collected and washed with the same buffer twice. The washed pellet was subsequently extracted with 5 volumes of buffer B (0.45 mM KCl, 5 mM β-mercaptoethanol (BME), 0.2 M Mg $(CH_3COO)_2$, 1 mM ethylene glycol bis (β-aminoethyl ether) N,N,N',N'- tetraacetic acid (EGTA), and 20 mM Tris-maleate, pH, 6.8). Adenosine 5'-triphosphate (ATP) was added to a final concentration of 15 mM. The mixture was kept for 1 h with stirring on ice and then centrifuged at 10,000×g for 15 min. Pellets were collected for actin preparation. Twenty five volumes of 1 mM $NaHCO_3$ was slowly added to the

supernatant and kept on ice for 30 min. The precipitate was recovered by centrifugation at 12,000×g for 15 min. The pellet was resuspended with 5 volumes of buffer C (0.50 M KCl, 5 mM BME, and 20 mM Tris-HCl, pH7.5) and homogenized for 30 s. The solution was kept on ice for 10 min and $MgCl_2$ was added to a final concentration of 10 mM. Myosin was obtained by ammonium precipitation at 40-50 % saturation. The myosin pellet was kept at -40 °C and used throughout the study. The purity of extracted myosin was estimated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) with densitometric analysis (Lab works Version 4.0,UVB Ltd., New York, USA). Before used, myosin pellet was dissolved in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0 and dialyzed against 100 volumes of the same solution. Dialyzed myosin was clarified by centrifugation at 10,000×g for 15 min and used as myosin solution. Protein concentrations were determined by Lowry and others (1951).

Actin Preparation

Actin pellet was added with buffer D (0.80 M KCl, 5 mM BME, 20 mM Tris-HCl, pH 7.5) and stirred for 30 min before centrifugation at 10,000×g for 15 min. The pellet was collected and added with 5 volumes of 2 mM $NaHCO_3$. The mixture was stirred on ice for 1 h and centrifuged at 75,000×g for 1 h. The supernatant was used as actin preparation. Actin was dialyzed against 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0. Dialyzed actin solution was concentrated by ultrafiltration using molecular weight-cut-off 10,000 Da membrane (Viva flow 50, Vivascience Sartorius AG, Goettingen, Germany). Purity of actin was estimated using SDS-PAGE and densitometric analysis.

Turbidity measurement

Myosin (3 mg/mL) and actin (1.5 mg/mL) solutions were solubilized in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0 containing 0, 10, 30, 50 and 100 mM $CaCl_2$. Turbidity at 25 and 40 °C were monitored at 350 nm using UV/VIS 916 spectrophotometer (*GBC Scientific Equipment, Ltd., Victoria, Australia.*) equipped with a circulating water bath set at either 25 or 40 °C. Turbidity changes at 25 and 40 °C were monitored at each time interval for 4 and 2 h, respectively.

Aggregation of TB myosin and actin

Myosin (3.2 mg/mL) and actin (1 mg/mL) solutions containing 0-100 mM $CaCl_2$ were incubated at either 25 or 40 °C for 4 and 2 h, respectively. Subsequently, samples were centrifuged at 84000×g for 1 h (XL-100 Ultracentrifuge, Beckman instruments, Inc., California, USA) to precipitate large aggregates. Protein concentrations in supernatants were determined by dye binding method due to interference of $CaCl_2$ with Lowry method (Bradford 1986). Bovine serum albumin (BSA) was used as a standard. Remaining proteins (%) was expressed as a ratio of protein remained in the supernatant at any $CaCl_2$ concentrations to that of sample without $CaCl_2$ at 4 °C.

Surface hydrophobicity (S_o)

Changes of S_o were monitored using 1-anilino-8-napthalenesulfonate (ANS) according to the method of Li-Chan and others (1985) with slight modifications. Myosin and actin were diluted to various protein concentrations: 0, 0.125, 0.25, 0.5 and 1 mg/mL in the presence of 0-100 mM $CaCl_2$ and incubated at either 25 or 40 °C for 4 and 2 h,

respectively. To 2.0 mL of diluted myosin and actin, 10 μL of 10 mM ANS dissolved in 20 mM Tris-maleate (pH 7.0) was added. Fluorescence intensity (FI) was measured using a RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. Blanks were prepared without protein solution. The regression slope between FI and protein concentrations (%) was calculated as S_o ANS.

Total sulfhydryl groups (SHs)

Total SH groups of myosin and actin were determined using 5,5'-dithiobis(2-nitrobenzoic acid), (DTNB). Myosin (3 mg/mL) and actin (1 mg/mL) solutions containing 0, 10, 30, 50 and 100 mM $CaCl_2$ were incubated at 25 and 40 °C for 4 and 2 h, respectively. Then, 1.5 mL of 0.2 M Tris-HCl (pH 6.8) containing 8 M urea, 2 % SDS and 10 mM EDTA was added. Subsequently, 0.2 mL of 0.1% DTNB solution were added to all samples before incubated at 40 °C for 15 min and absorbance at 412 nm was measured. Total SH content was calculated using molar extinction of 13600 M^{-1} cm^{-1} for myosin (Ellman, 1959). Molar extinction of actin used for the calculation was 12508 M^{-1} cm^{-1} ,which was obtained using standard L-cysteine. Blanks were performed without protein solution.

Ca-ATPase activity

Ca-ATPase activity of myosin was estimated using the method described by MacDonald and others (1994). The reaction was carried out at 1.5 mg of myosin, 17 mM Tris-maleate, pH 7.0 at 0-200 mM $CaCl_2$ concentrations. The mixtures were incubated at

25 °C for 5 min. ATP was added to final concentration of 0.67 mM and samples were incubated for 10 min. To stop the reaction, chilled TCA was added to final concentration of 5 % and samples were centrifuged at 3,000×g for 10 min. The supernatant was collected for inorganic phosphate (Pi) determination using KH_2PO_4 as a standard. Ca-ATPase activity was expressed as μmole of Pi/mg protein/min at 25 °C.

Circular dichroism (CD)

The effect of $CaCl_2$ on secondary structural changes of myosin and actin were analyzed using CD measurement. Myosin and actin were dissolved in 0.6 M NaCl, 20 mM Tris-HCl, pH 7.0 due to strong UV absorption of Tris-maleate buffer. Myosin and actin solutions (0.25 mg/mL) containing $CaCl_2$ (0-100 mM) were incubated at either 25 or 40 °C for 4 and 2 h, respectively. Samples were scanned at far UV (195-280 nm) using a JASCO PS-150J spectropolarimeter (Jasco spectroscopic Co, Ltd., Tokyo, Japan) equipped with a circulating water bath set at each respective incubating temperature. CD spectra of samples without incubation were also measured at 4 °C. The instrument was calibrated using (1*S*)-(+)-10-camphorsulfonic acid (CSA). The circular quartz cuvette (0.02 cm path length) was used. Resolution was set at 1 nm, bandwidth was 2 nm, sensitivity 50 mdeg, response 2 s and scanning speed was 50 nm/min. Molar mean ellipticity [θ] and α-helical content from [θ] at 222 nm was calculated according to Ogawa and others (1993).

Results and Discussion

Effect of $CaCl_2$ on aggregation of myosin and actin

Purity of myosin was estimated to be 90-91%. Four minor contaminated bands with Mw of 43, 37, 34, and 27 kDa were observed in myosin (Figure 1). The 43 and 37 kDa bands were possibly actin and tropomyosin, respectively. TB actin showed molecular weight of 43 kDa and exhibited high purity (>97%). Ca-ATPase activity of purified myosin at 3.3 mM $CaCl_2$ was 0.220 μmole Pi/mg protein/min.

Low concentrations of $CaCl_2$ (0-50 mM) did not affect turbidity of myosin solution incubated at 25 °C for 4 h, while 100 mM $CaCl_2$ increased turbidity of myosin at 25 °C (Figure 2a). Gill and others (1992) demonstrated that an increase in turbidity of heated fish myosin solution was the direct result of formation of myosin aggregates. Therefore, aggregation of TB myosin was enhanced at 25 °C in the presence of 100 mM $CaCl_2$. Moreover, aggregation of TB myosin occurred to a greater extent at 40 °C than at 25 °C (Figure 2b). Turbidity of actin solution incubated at 25 °C sharply increased with $CaCl_2$ concentration, especially at 50 and 100 mM $CaCl_2$ (Figure 2c). Aggregation of actin dramatically increased when incubated in the presence of 10 mM $CaCl_2$ at 40 °C. However, a further increase of $CaCl_2$ from 30 to 100 mM did not increase actin aggregation (Figure 2d). Actin appeared to aggregate to a greater extent than myosin even at lower protein concentration.

Large protein aggregates tend to precipitate under high centrifugal force. TB myosin incubated at 25 °C did not form large aggregates that could be precipitated under centrifugation at any studied levels of $CaCl_2$ (Figure 3a). In contrast, precipitation of myosin was observed when incubated at 40 °C and the extent of aggregation appeared to increase with $CaCl_2$ (10-100 mM). Based on turbidity results, TB myosin appeared to form soluble aggregates at 25 °C, while large aggregates were formed at 40° C. Since

denaturation temperature (Td) of TB actomyosin was about 35 °C (Yongsawatdigul and Park 2003), TB myosin subjected to 40 °C could unfold and re-associate to form aggregates. Addition of 10-100 mM $CaCl_2$ further promoted myosin aggregation. These myosin aggregates were unlikely to be resulted from the catalytic reaction of endogenous TGase because the enzyme was mainly removed during extensive washing and precipitation steps of myosin purification. This was evident by the absence of nondisulfide covalent cross-links of myosin when incubated at either 25 or 40 °C to induce endogenous TGase (data not shown).

Actin readily precipitated even at 4 °C without $CaCl_2$ (Figure 3b). The extent of actin aggregation also increased with temperature. Similar to myosin, actin aggregation was also enhanced by $CaCl_2$. The extent of aggregation monitored by ultracentrifugation corresponded with changes of turbidity. Moreover, aggregation of actin was completely attained when incubated at 10-100 mM $CaCl_2$ at 40 °C for 2 h (Figure 3b). These results indicated that Ca^{2+} induced aggregation of TB myosin and actin when incubated at 40 °C to a greater extent than at 25 °C.

Effect of $CaCl_2$ on surface hydrophobicity (S_o ANS) of myosin and actin

S_o ANS of myosin slightly increased with $CaCl_2$ concentration at all studied temperatures (Figure 4a), indicating that Ca^{2+} promoted the unfolding of myosin. It was noted that changes in S_o ANS of myosin incubated at 25 °C for 4 h were similar to those incubated at 40 °C, but higher than those at 4 °C (Figure 4a). It should be noted that TB myosin only form soluble aggregates at 25 °C (Figure 3a). Incubation of myosin at 25 °C was far below Td of tropical fish myosin, which has been reported to be 37- 43 °C (Sano

and others 1990). Limited unfolding of myosin at 25 °C would restrict intermolecular entanglement via any interactions, resulting in formation of soluble aggregates, rather than large aggregates.

At 40 °C, myosin molecules underwent partial unfolding due to thermal denaturation. The partial unfolded molecules exposed the previously buried hydrophobic groups to the aqueous environment, which subsequently re-associated via hydrophobic interactions. As a result, large aggregate formation at 40 °C was evident (Figure 3a). Hydrophobic interactions of unfolded molecules would reduce ANS-binding capacity. This explained why S_o ANS at 40 °C was comparable to that at 25 °C in spite of the greater extent of unfolding occurred at 40 °C. Lanier (2000) suggested that hydrophobic interactions participated in gelation during setting. Thus, Ca^{2+} ion induced the unfolding of myosin, which could in turn enhance hydrophobic interactions among myosin molecules during setting.

S_o ANS of actin also increased with $CaCl_2$ concentration and exhibited higher values than those of myosin at all temperature studied (4, 25 and 40 °C) (Figure 4b). This may be partly due to the inactivation of actin by EGTA used in actin extraction (Turoverov and others 1999). The inactivated actin tended to expose hydrophobic clusters on the surface and showed high affinity to hydrophobic probes (Lehrer 1972). Moreover, existence of large hydrophobic groups on the surface led to self-association of actin monomers (Mazhul and others 2003). Thus, the greater extent of aggregation and exposure of surface hydrophobicity was observed in actin. Transformation of native G-actin to inactivated form resulted in partial unfolded structure, which was more prone to

denaturation. Our study showed that Ca^{2+} induced more open structure of inactivated actin, leading to the aggregate formation via hydrophobic interactions.

Effect of $CaCl_2$ on total SH groups of myosin and actin

Total SH groups of myosin and actin in the absence of $CaCl_2$ at 4 °C were $\approx 6 \times 10^{-5}$ and 5×10^{-5} mole /g protein, respectively. When myosin was incubated at 40 °C for 2 h, total SH groups decreased to $\approx 4.8 \times 10^{-5}$ mole /g protein as a result of thermal denaturation. In the presence of Ca^{2+} ion, SH groups of myosin incubated at all studied conditions continuously decreased as $CaCl_2$ concentration increased (Figure 5a). A marked decrease in SH group was observed when incubated at 40 °C for 2 h. The similar trend was also observed in actin (Figure 5b). These results indicated that Ca^{2+} induced the formation of disulfide linkages of both myosin and actin when incubated at 40 °C. The unfolding of myosin and actin induced by $CaCl_2$ resulted in an exposure of free SH groups, which subsequently underwent disulfide interchanges. Similar effect of $CaCl_2$ on the formation of disulfide linkages and hydrophobic interactions were also found in α-crystallin molecules (Valle and others 2002). It should be noted that the extent of disulfide bond formation at 40 °C was greater than that at 25 °C (Figure 5a). It was, therefore, speculated that disulfide bond might be partly responsible for aggregation of myosin set at 40 °C. Addition of $CaCl_2$ to fish protein paste induced hydrophobic interactions and disulfide linkages of myosin and actin at 40 °C to a greater extent than at 25 °C. Besides ε-(γ-glutamyl) lysyl isopeptide bonds catalyzed by Ca^{2+}- dependent endogenous TGase, hydrophobic interactions and disulfide linkages could be involved during setting of fish protein.

When Ca^{2+} ion was not added, setting phenomenon at 25 °C was not observed in surimi made from tropical fish (Kamath and others 1992; Klesk and others 2000). The existing explanation was that tropical fish exhibited higher thermal stability that limited the exposure of reactive groups on myosin molecule for TGase catalytic reaction. However, Yongsawatdigul and others (2002) reported the setting of TB surimi at 25 °C when 0.1% $CaCl_2$ (≈ 10 mM) was added. Our study revealed that addition of Ca^{2+} ion (≥10 mM) increased more exposure of hydrophobic amino groups and more disulfide linkages of myosin and actin, which subsequently contributed to setting phenomenon of TB at 25 °C.

Effect of $CaCl_2$ on Ca-ATPase activity of myosin

Ca-ATPase activity of myosin slightly increased and reached the maximum at 50 mM $CaCl_2$ (Table 1). Further increase of $CaCl_2$ concentration dramatically reduced Ca-ATPase activity. Ca-ATPase activity at 200 mM $CaCl_2$ was about 36 % of that at 50 mM $CaCl_2$. High level of $CaCl_2$ (> 50 mM) induced conformational changes of globular head of myosin, resulting in a decrease of Ca-ATPase activity. The exposure of hydrophobic and changes of total SH groups at $CaCl_2$ < 50 mM was likely to occur at myosin rod, while both globular and rod portions underwent such changes at high $CaCl_2$ concentration (> 50 mM).

Binding of Ca^{2+} to anionic sites on protein structure can induce the unfolding. These binding interactions prevent salt exclusion from protein structure and decrease preferential hydration of salts, resulting in salting-in and destabilization of protein structure (Arakawa and Timasheff 1984). Myosin contained negative charges at pH 7

because pI of myosin is around 4.8-6.2 (Stefansson and Hultin 1994). Thus, the ionic interactions between Ca^{2+} and negatively charged myosin might be responsible for disturbance of native myosin molecules. Ca^{2+} also induced aggregation of β-lactoglobulin (theoretical net charge, Z = -8) by selective binding to carboxylated anions (Simons and others 2002). For myosin, most negative charges are located at myosin rod (Z = - 34 to -52) and followed by myosin light chains (Z = -6 to -27), while globular head myosin has positive charges (Z = 6 to 16) (Bechet and Albis 1989). Therefore, Ca^{2+} was more likely to bind to myosin rod than the globular head. For this reason, the rod portion was more susceptible to conformational changes induced by Ca^{2+} ion.

Effect of $CaCl_2$ on CD spectra of myosin and actin

CD spectra in far UV region of myosin showed predominant α-helix structure (Figure 6a). $CaCl_2$ promoted the loss of secondary structure of both myosin and actin even at 4 °C (Figure 6a,b). The helical content of myosin at 4 °C without $CaCl_2$ was 71.2% and decreased to 51.4% in the presence of 100 mM $CaCl_2$ (Figure 7a). The helical content of myosin incubated at 25 °C for 4 h was slightly decreased with increasing $CaCl_2$ concentration. In contrast, $CaCl_2$ markedly decreased helical content of myosin incubated at 40 °C for 2 h (Figure 7a). Both thermal energy and $CaCl_2$ synergistically contributed to unfolding of myosin at 40 °C, leading to considerable loss of helical structure. Ogawa and others (1995) reported that loss of helical structure of fish actomyosin was a pre-requisite to initiate setting. Therefore, addition of $CaCl_2$ accompanied by incubating at 40 °C enhanced myosin unfolding, which subsequently

resulted in a higher degree of hydrophobic interactions and formation of disulfide linkages.

Low helical content (28.73 %) was observed in actin at 4 °C (Figure 7b). Nagy et al. (1972) reported that actin contained 30% α-helix structure, 10% of β-sheet and the remaining residues did not appear to contribute to the optical activity (1972). α-Helical content of actin decreased when incubated at 25 °C in the presence of 10 mM $CaCl_2$. However, further increase of $CaCl_2$ from 30 to 100 mM did not further decrease helical content of actin. Moreover, α-helical structure of actin incubated at 40 °C for 2 h was completely destroyed at10 mM $CaCl_2$. These results suggested that $CaCl_2$ at ≥10 mM also induced the changes of secondary structure of actin.

Conclusions

Ca^{2+} ion induced conformational changes of TB myosin and actin leading to partial unfolding and exposure of hydrophobic amino acids. The unfolded molecules subsequently aggregated via hydrophobic interactions and disulfide linkages when incubated at either 25 or 40 °C. Such interactions could be important in gel-forming of TB during setting. Thus, $CaCl_2$ did not only enhance gelling properties of TB myosin through activating endogenous TGase but also directly induce conformational changes of myosin and actin, promoting hydrophobic interactions and disulfide linkages of "set" gel.

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Figure legends

Figure 1. SDS-PAGE patterns of TB myosin and actin. S= molecular weight standard, MI= TB mince, MY_1 and MY_2 = myosin from lot 1 and lot 2, respectively. AC_1 and AC_2 = actin from lot 1 and lot 2, respectively. MHC= myosin heavy chain, LC = myosin light chains.

Figure 2. Effect of $CaCl_2$ on turbidity of TB myosin and actin incubated at either 25 or 40 °C in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0: myosin at 25 °C (a), myosin at 40 °C (b), actin at 25 °C (c), and actin at 40 °C (d).

Figure 3. Remaining protein contents of TB myosin (a) and actin (b) at varied $CaCl_2$ concentration after centrifugation at 78,000 ×g for 1 h.

Figure 4. Effect of $CaCl_2$ on the changes in S_o ANS of TB myosin (a) and actin (b) in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0.

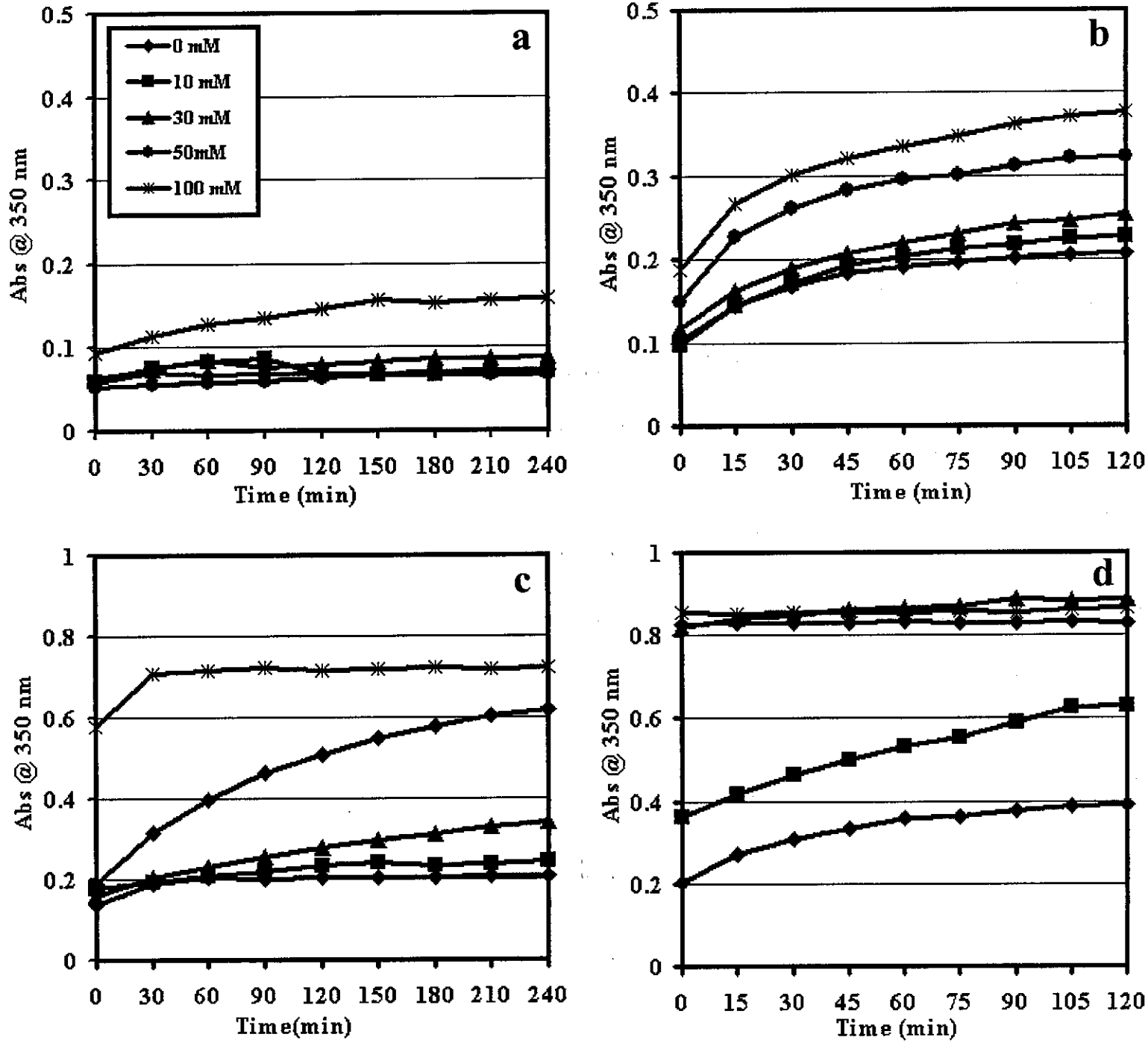
Figure 5 Effect of $CaCl_2$ on the changes in total SH groups of TB myosin (a) and actin (b) in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0.

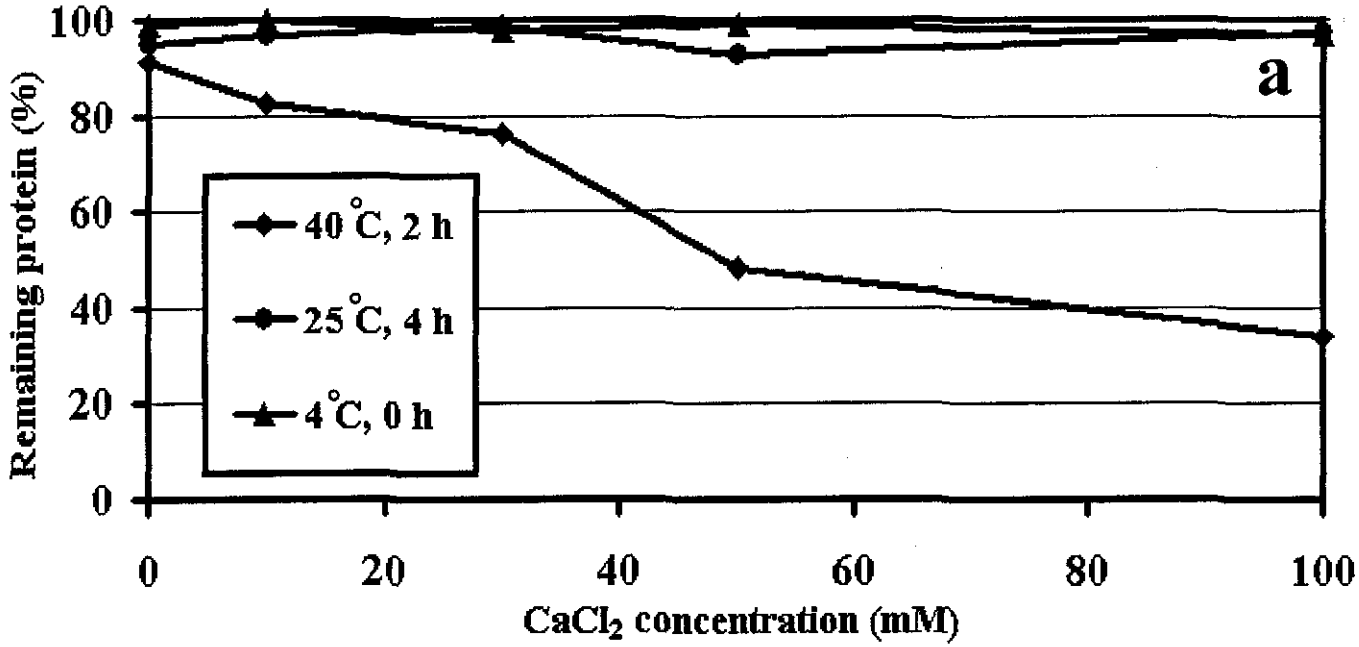
Figure 6 Effect of $CaCl_2$ on CD spectra of TB myosin (a) and actin (b) in 0.6 M NaCl, 20 mM Tris-HCl, pH 7.0 at 4 °C.

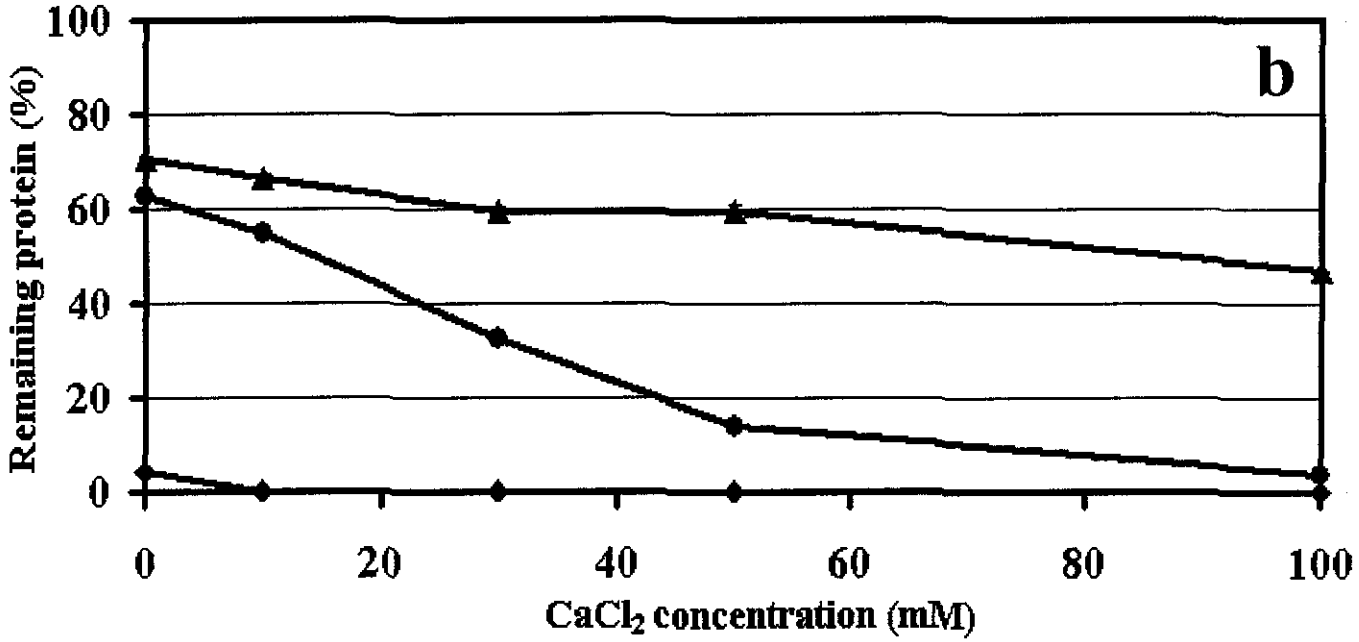
Figure 7 Effect of $CaCl_2$ on the changes in α-helical contents of TB myosin (a), and actin (b).

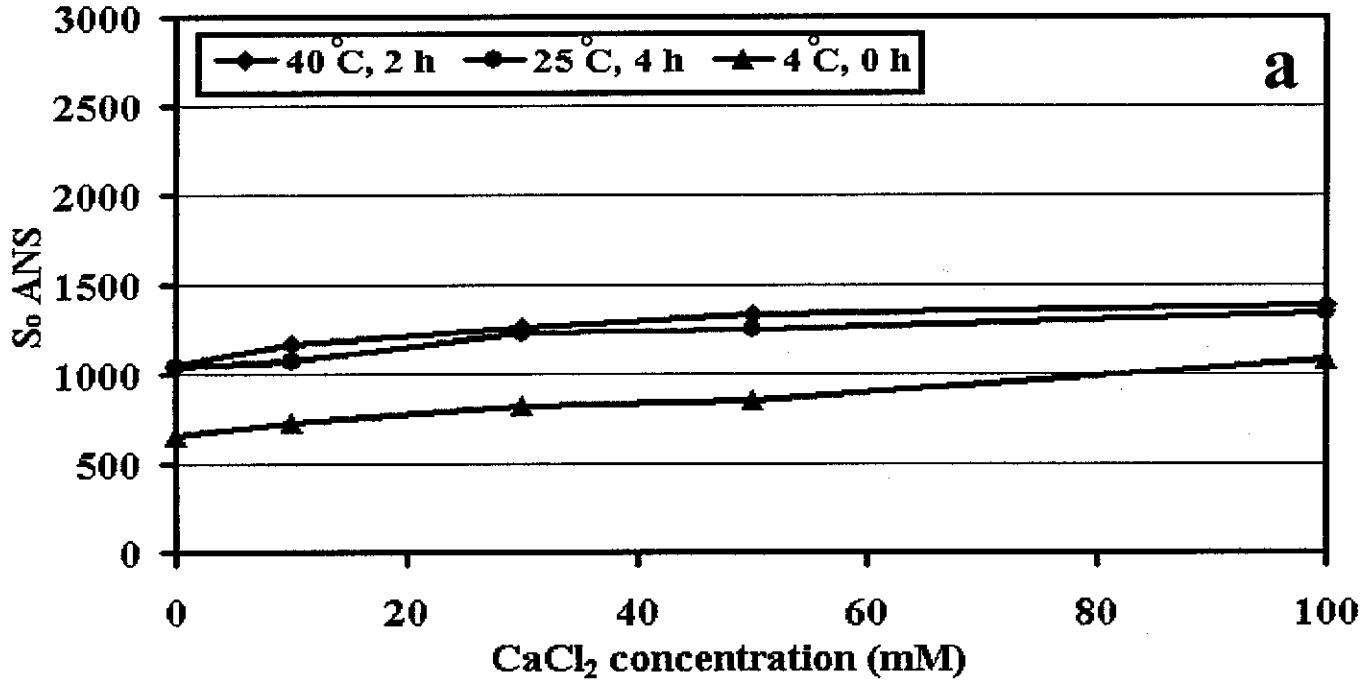
Table 1. Ca -ATPase activity of TB myosin determined at various $CaCl_2$ concentrations at 25 °C.

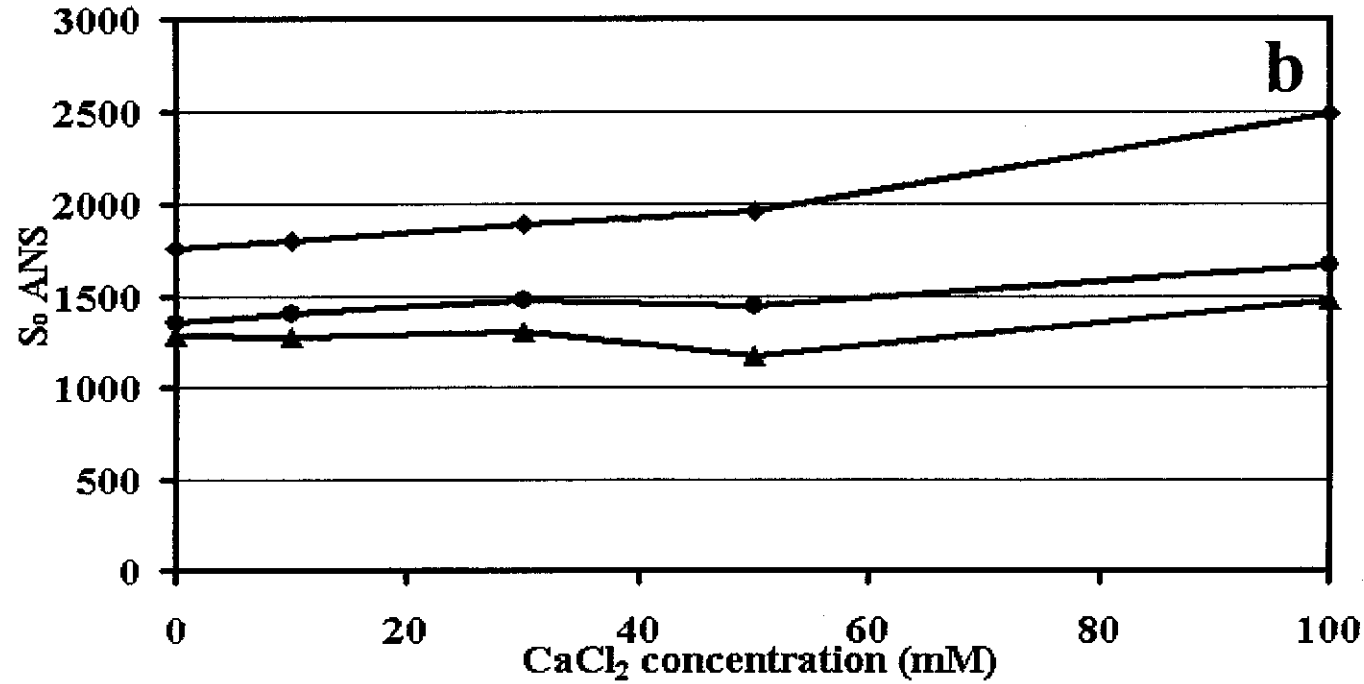
$CaCl_2$ concentration (mM)	Ca-ATPase activity (μ mole Pi/mg protein/min)
0	0
3.3	0.274 ± 0.025
10	0.297 ± 0.013
30	0.313 ± 0.023
50	0.299 ± 0.010
100	0.229 ± 0.011
150	0.185 ± 0.020
200	0.106 ± 0.019

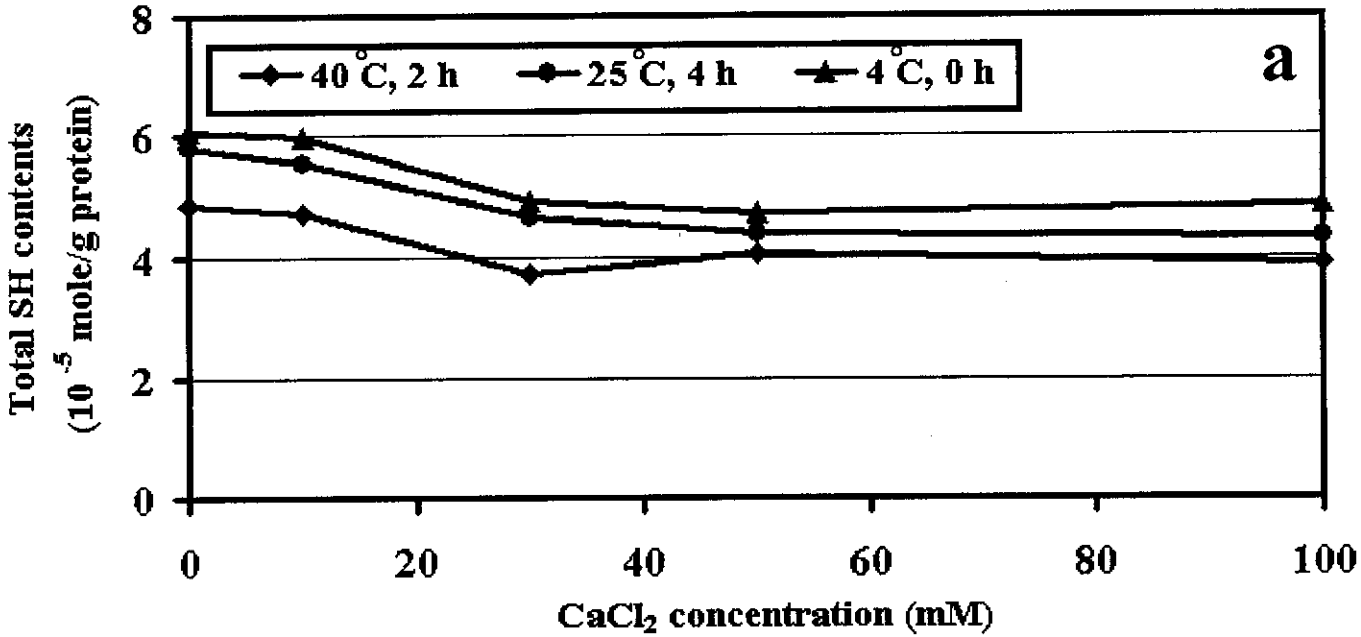

a
0 mM
10 mM
30 mM
50mM
100 mM
Abs @ 350 nm
Time (min)
b
Abs @ 350 nm
Time (min)
c
Abs @ 350 nm
Time(min)
d
Abs @ 350 nm
Time (min)

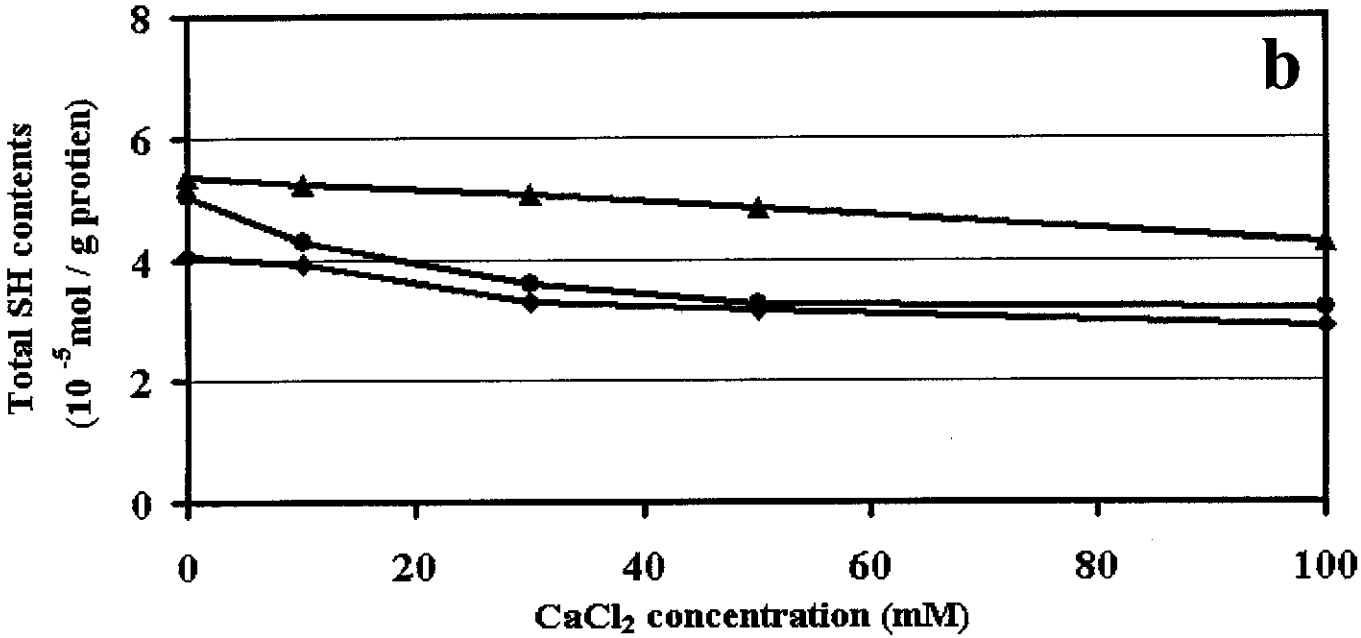

a
Remaining protein (%)
100
80
60
40
20
0
40 °C, 2 h
25 °C, 4 h
4 °C, 0 h
0
20
40
60
80
100
CaCl2 concentration (mM)

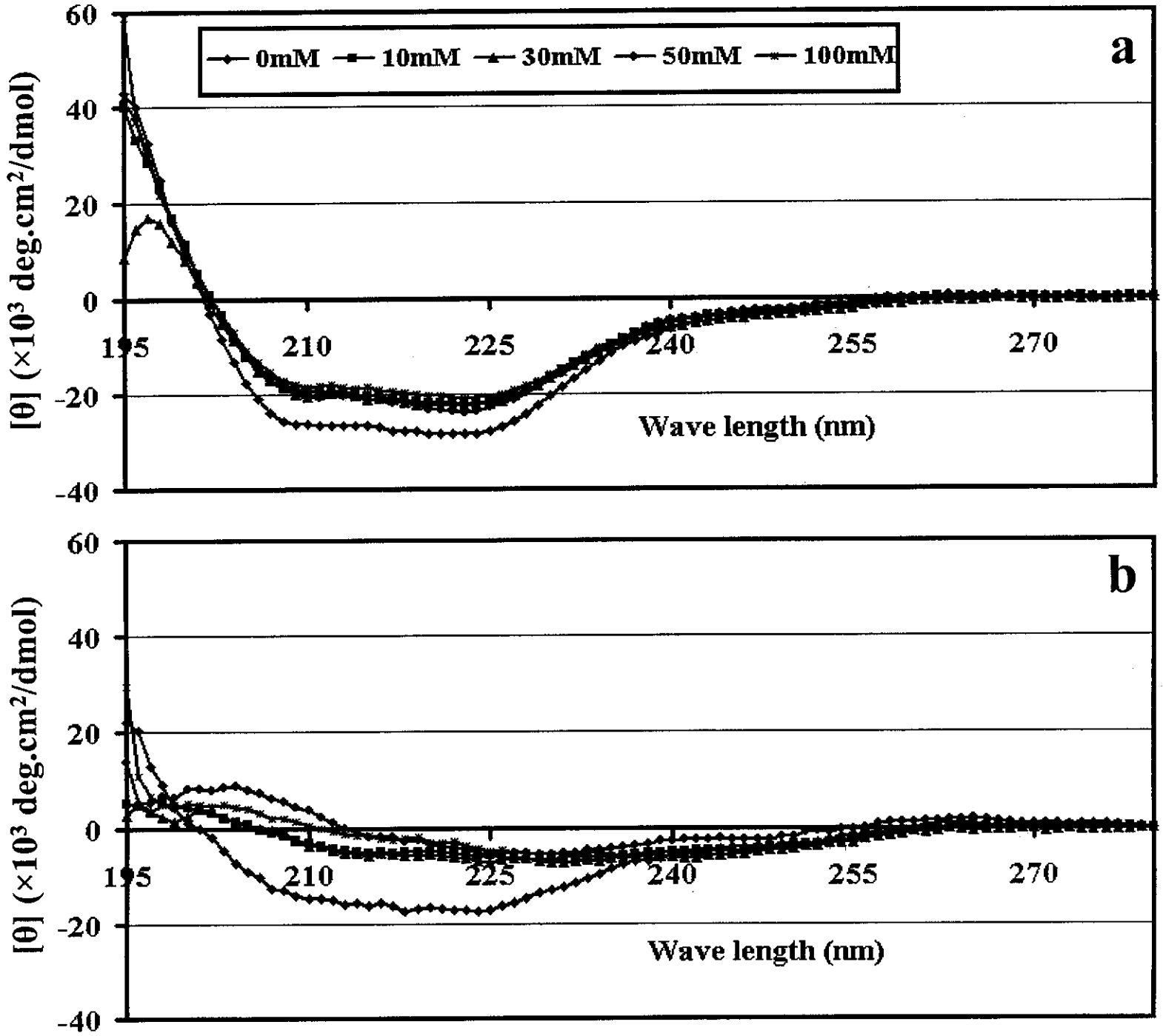

b
Remaining protein (%)
100
80
60
40
20
0
0
20
40
60
80
100
CaCl2 concentration (mM)

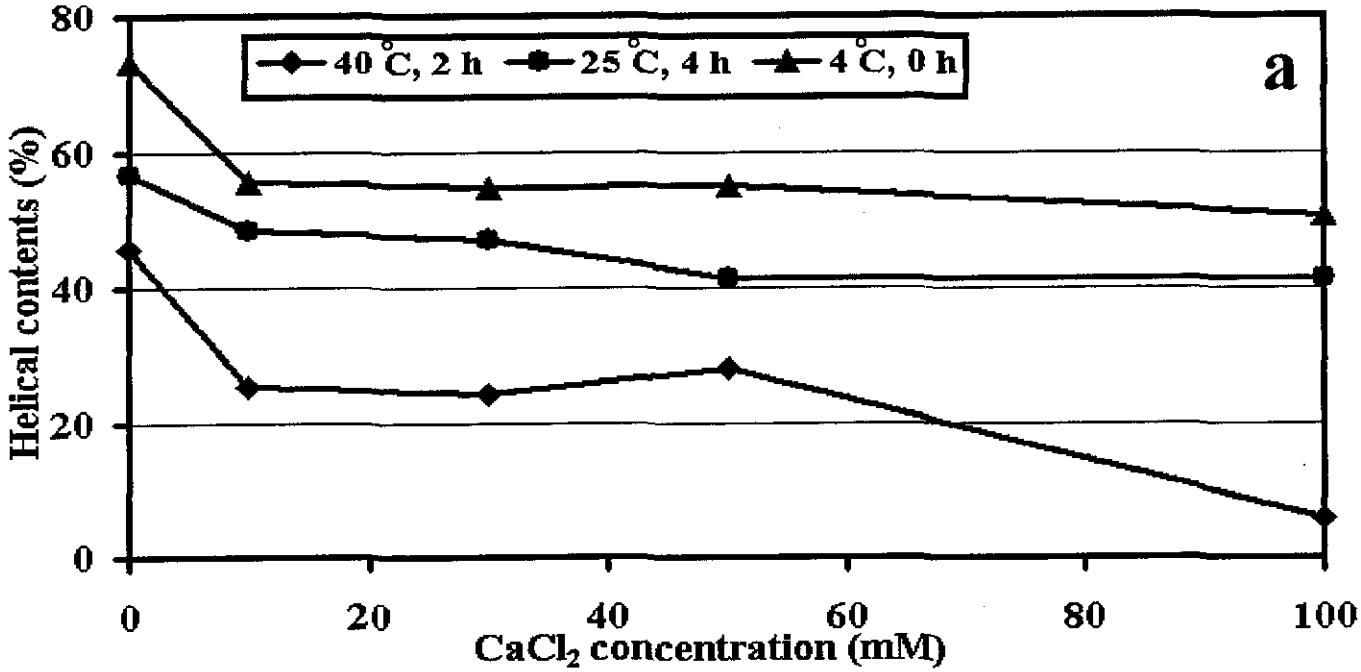

40 °C, 2 h
25 °C, 4 h
4 °C, 0 h
a
3000
2500
2000
1500
1000
500
0
S0 ANS
0
20
40
60
80
100
CaCl2 concentration (mM)

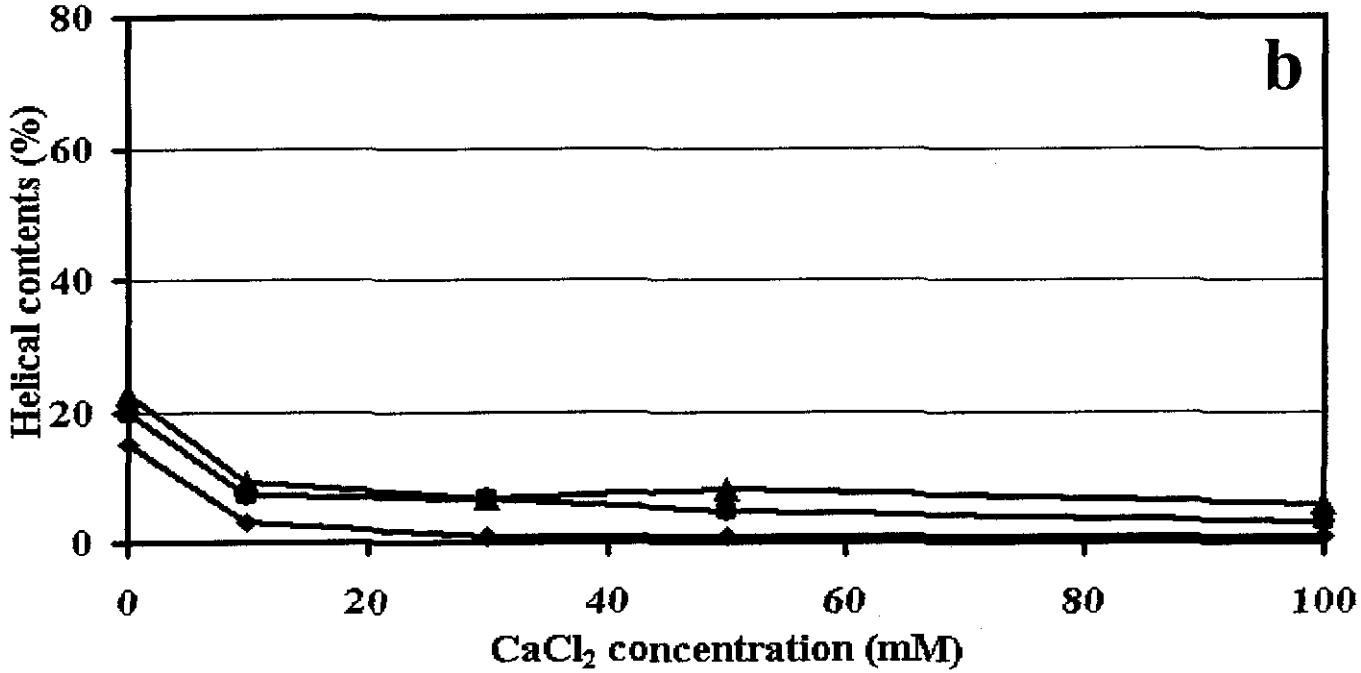

b
3000
2500
2000
1500
1000
500
0
S0 ANS
0
20
40
60
80
100
CaCl2 concentration (mM)


40 °C, 2 h
25 °C, 4 h
4 °C, 0 h
a
Total SH contents
(10 -5 mole/g protein)
0
2
4
6
8
0
20
40
60
80
100
CaCl2 concentration (mM)


b
Total SH contents
(10 -5 mol / g protien)
0
2
4
6
8
0
20
40
60
80
100
CaCl2 concentration (mM)


0mM
10mM
30mM
50mM
100mM
a
b
[θ] (×10³ deg.cm²/dmol)
Wave length (nm)
60
40
20
0
-20
-40
195
210
225
240
255
270


40 °C, 2 h
25 °C, 4 h
4 °C, 0 h
a
Helical contents (%)
0
20
40
60
80
0
20
40
60
80
100
CaCl2 concentration (mM)


b
Helical contents (%)
0
20
40
60
80
0
20
40
60
80
100
CaCl2 concentration (mM)